USE OF PHENYLACETYL GROUP FOR PROTECTION OF THE LYSINE N^{ε}-AMINO GROUP IN SYNTHESIS OF PEPTIDES*

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N^{ε}-Phenylacetyllysine was used in the synthesis of deamino-lysine-vasopressin. The phenylacetyl protecting group is stable under conditions of peptide synthesis and can be removed by action of penicillin amidohydrolase (E.C.3.5.1.11) without damaging the built peptide chain.

Removal of protecting groups by enzymes is advantageous for their selective action and mild reaction conditions, particularly in cases of higher and structurally more complicated peptides.** Enzymes have been hitherto used mainly for removal of carboxyl-protecting groups. Alkyl esters were cleaved by trypsin[2], chymotrypsin[2-6] and carboxypeptidase Y (ref.[7]).

Also arginine has been used as the "protecting group" which was cleaved off by carboxypeptidase B (ref.[8,9]). Protection of an amino group was accomplished by benzoylphenylalanine[10] and benzyloxycarbonylarginine[11], removable by chymotrypsin and trypsin, respectively. Both these methods are of limited applicability: the first cannot be used if the synthesized peptide contains an aromatic amino acid, the second if it contains a basic amino acid. This paper describes use of the phenylacetyl group for protection of the lysine ε-amino group in the peptide synthesis and its removal by action of penicillin amidohydrolase (E.C.3.5.1.11). This enzyme, isolated from *E. coli*, cleaves off phenylacetic acid from penicillin G to give 6-aminopenicillanic acid[12-15]. The phenylacetyl group is removed also from amino acids[16] and several amines[17]. The stereospecificity of penicillin amidohydrolase was used in configurational correlations of amines[18,19] and for the resolution of S-benzyl-D,L-penicillamine[20].

N^{ε}-Phenylacetyllysine (*Ia*) was prepared by acylation of the copper complex of lysine. The derivative *Ia* is stable towards hydrogen bromide in acetic acid as well

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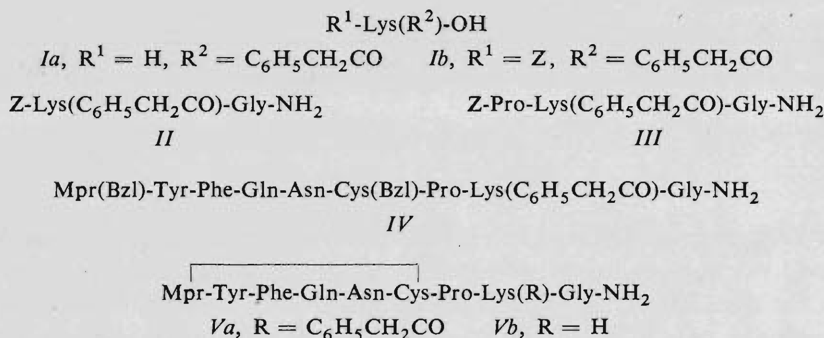
** The amino acids used in this paper are of the L-series. The nomenclature and symbols obey the published recommendations[1]; Mpr denotes the β-mercaptopropionic acid residue.

as sodium in liquid ammonia. The N^{α}-benzyloxycarbonyl derivative *Ib* was condensed with glycine amide to give the dipeptide *II*. After removal of the benzyloxycarbonyl group with hydrogen bromide in acetic acid the resulting dipeptide-amide was acylated with benzyloxycarbonylproline in the presence of dicyclohexylcarbodiimide, affording the protected tripeptide *III*. The benzyloxycarbonyl group was removed in the same manner as described for the compound *II* and the tripeptide-amide was acylated with S-β-benzylmercaptopropionyl-tyrosyl-phenylalanyl-glutaminyl-asparaginyl-S-benzylcysteine azide, prepared from the corresponding hydrazide[21]. The resulting peptide *IV* was reduced with sodium in liquid ammonia, the disulfide bond



was formed by oxidative cyclization and the protected cyclopeptide *Va* was purified by countercurrent distribution and gel filtration. The phenylacetyl protecting group was removed from the ε-amino group of lysine by action of penicillin amidohydrolase and the obtained deamino-lysine-vasopressin *Vb* was purified by free-flow electrophoresis. The optimal incubation time for the transformation of compound *Va* into *Vb* was found by continuous checking of the pressoric and uterotonic activity. The found pressoric activity of compound *Vb* (180 I.U./mg) was somewhat higher than that given in the literature[22-24] (125, 126 and 132 I.U./mg). This discrepancy is obviously due to differently performed biological tests; the uterotonic activity and the optical rotation of our sample agreed very well with the published values. We determined also the biological activity of [8-N^{ε}-phenylacetyllysine]deamino-vasopressin (*Va*) (uterotonic activity in Mg^{++}-free solution 99 I.U./mg, galactogogic activity 106 I.U./mg and pressoric activity 3·8 I.U./mg).

Model experiments in which as substrates we used compound *II* and *III* (without the benzyloxycarbonyl group) have shown that a technical penicillin amidohydrolase preparation removes quantitatively the phenylacetyl group in 90 min whereas the peptide is not affected by the enzyme even after 21 h.

EXPERIMENTAL

The melting points were determined on a Kofler block and are uncorrected. Analytical samples were dried for 24 h at room temperature and 150 Pa. Thin-layer chromatography was performed on silica gel (Silufol, Kavalier, Czechoslovakia) using the following systems: S1 2-butanol–98% formic acid–water (75 : 13·5 : 11·5), S2 2-butanol–25% aqueous ammonia–water (85 : 7·5 : 7·5), S3 1-butanol–acetic acid–water (4 : 1 : 1), S4 1-butanol–acetic acid–pyridine–water (15 : 3 : 10 : 6), S7 1-butanol–acetic acid–ethyl acetate–water (1 : 1 : 1 : 1), S11 chloroform–methanol–acetic acid–water (32 : 8 : 2 : 1·2). Paper electrophoresis was carried out in a moist chamber in 1M acetic acid (pH 2·4) or in a pyridine–acetate buffer (pH 5·7) on a paper Whatman 3MM, 20 V/cm, 60 min. Spots were detected by ninhydrin or by the chlorination method. Amino acid analyses were performed after hydrolysis of the samples in 6M-HCl for 20 h at 105°C in ampoules, evacuated at 150 Pa, using an automatic analyzer (type 6020, Development Workshops, Czechoslovak Academy of Sciences, Prague). Reaction mixtures were taken down at 30—40°C (bath) on a rotatory evaporator under diminished pressure (water pump); dimethylformamide-containing solutions were evaporated using an oil pump (150 Pa). Optical rotations were measured on a Perkin–Elmer 141 MCA instrument.

Isolation and Purification Methods

Countercurrent distribution was carried out in an all-glass apparatus (Steady State Distribution Machine, Quickfit & Quartz, Stone, Staffordshire, England) with possibility of transfers of the upper as well as the lower phases; system 2-butanol–0·05% aqueous acetic acid. The peptide material was located using the Folin–Ciocalteau reaction. Free-flow electrophoresis was performed on a previously described[25,26] apparatus; the purification was carried out in 0·5M acetic acid at 2 600 V; the peptide material was detected by the absorption at 280 nm. Gel filtration was performed on columns packed with Sephadex LH-20 (200 × 1 cm, flow rate 10 ml/h, dimethylformamide) or with Bio-Gel P-4 (140 × 2·5 cm, flow rate 10 ml/h, 3M acetic acid); detection at 280 nm.

Penicillin Amidohydrolase (E.C.3.5.1.11)

The enzyme employed was a technical preparation isolated[27] from *E. coli* cells. Its activity, determined on penicillin G as substrate[28], was 8·5 μmol mg^{-1} min^{-1} of liberated 6-aminopenicillanic acid.

N^{ε}-Phenylacetyllysine (*Ia*)

Copper carbonate dihydroxide (freshly prepared from 12·5 g of $CuSO_4.5\,H_2O$ and 9 g of $NaHCO_3$) was added to a boiling solution of lysine hydrochloride (9·2 g) in water (125 ml) in the course of 30 min. The mixture was refluxed for 2 h with intermittent stirring. The insoluble portion was filtered off and the filtrate was treated (portionwise) with phenylacetyl chloride (13 ml) and 2M-NaOH, the pH of the solution being kept at 10. After 30 min the precipitated copper complex was collected on filter, washed with water and ether and suspended in hydrochloric acid (pH 2). The suspension was heated to 90°C, gaseous hydrogen sulfide was introduced for 1 h and the mixture was filtered with charcoal. The insoluble portion on the filter was washed with hydrochloric acid (pH 2), the filtrates were cooled to room temperature and the solution was adjusted to pH 7. After cooling, the separated material was filtered and crystallized from 3M acetic acid, affording 6·2 g (50%) of the product, m.p. 232—234°C; $[\alpha]_D$ +17·5° (*c* 0·9, 1M-HCl).

$E_{2.4}^{Gly}$ 0·62, $E_{5.7}^{Gly}$ 1·0; R_F 0·29 (S1), 0·26 (S3), 0·33 (S4). For $C_{14}H_{20}N_2O_3$ (264·3) calculated: 63·62% C, 7·63% H, 10·60% N; found: 63·79% C, 7·66% H, 10·51% N. Ninhydrin constant 61·0 (leucine 56·5); in amino acid analysis the peak begins in 129 min (corresponding time for tyrosine is 133 min).

Stability of the Phenylacetyl Group toward Sodium in Liquid Ammonia

N^{ε}-Phenylacetyllysine (264 mg) was treated for 30 seconds with sodium in liquid ammonia (50 ml), the excess sodium was removed with ammonium chloride, ammonia was evaporated *in vacuo*, the residue dissolved in water (5 ml) and filtered through a column of Dowex 50 W (H^+-form; 15 ml). The column was washed with water and the product eluted with 20% pyridine and 10% NH_4OH). The combined eluates were taken down, affording 250 mg (95%) of N^{ε}-phenylacetyllysine. No lysine was found by electrophoresis. According to amino acid analysis, the product contained less than 0·5% of lysine and its IR spectrum was identical with that of analytically pure N^{ε}-phenylacetyllysine.

N^{α}-Benzyloxycarbonyl-N^{ε}-phenylacetyllysine (*Ib*)

Benzyloxycarbonyl chloride (3 ml) and 1M-NaOH (to maintain pH 10) were added dropwise in the course of 30 min to a stirred slurry of N^{ε}-phenylacetyllysine (4 g) in 1M-NaOH (30 ml). The mixture was then stirred for 1 h, extracted with ether and the aqueous layer acidified (pH 3). The separated oil was taken up in ethyl acetate, the solution washed with water, dried over sodium sulfate and taken down, affording 6·1 g of an oil, R_F 0·30 (S2), 0·74 (S3), 0·69 (S4). The dicyclohexylammonium salt was crystallized from ethyl acetate; m.p. 134—135°C, $[\alpha]_D$ +2·4° (*c* 1, methanol), −3·3° (*c* 1, dioxane). For $C_{34}H_{49}N_3O_5$ (579·8) calculated: 70·44% C, 8·52% H, 7·25% N; found: 70·58% C, 8·45% H, 7·52% N.

N^{α}-Benzyloxycarbonyl-N^{ε}-phenylacetyllysyl-glycine Amide (*II*)

A solution of glycine amide hydrobromide (2·0 g) and N-ethylpiperidine (1·78 ml) in dimethylformamide (15 ml) was added to a solution of *Ib* (5·2 g) and 1-hydroxybenzotriazole (1·76 g) in dimethylformamide (15 ml). The mixture was cooled to −10°C, treated with dicyclohexylcarbodiimide (2·7 g), stirred at −5°C for 1 h and at room temperature for 16 h. Dimethylformamide was evaporated, the residue mixed with water and cooled to 0°C. The solid portion was filtered and washed with a saturated solution of sodium hydrogen carbonate, water, 0·5M-HCl and again with water. Crystallization from ethanol afforded 5·2 g (89%) of product, m.p. 155·5 to 156°C. R_F 0·67 (S1), 0·57 (S2), 0·66 (S3), 0·74 (S4); $[\alpha]_D$ −2·8° (*c* 0·3, dimethylformamide). For $C_{24}H_{28}N_4O_5$ (454·4) calculated: 63·42% C, 6·65% H, 12·32% N; found: 63·70% C, 6·80% H, 12·30% N.

Removal of phenylacetyl group by penicillin amidohydrolase: A 4M solution of hydrogen bromide in acetic acid (0·5 ml) was added to a solution of the dipeptide *II* (45 mg) in acetic acid (0·5 ml). After 30 min the mixture was mixed with ether and the solvent was decanted from the separated hydrobromide which was then dried. R_F 0·13 (S3), 0·45 (S4); $E_{5.7}^{His}$ 0·65, $E_{2.4}^{His}$ 0·68. A part of the product (2·6 mg) was dissolved in a 0·1M phosphate buffer (pH 7·5; 260 µl) and the solution was treated with a solution of penicillin-amidohydrolase (135 µl; the enzyme solution was prepared by dissolving 10 mg of the enzyme preparation in 1 ml of phosphate buffer and removing a small amount of solid by centrifugation) and with water (135 µl). The mixture was incubated at 37°C and the samples were taken after 90 min and 21 h. Both the samples consisted of a single compound of R_F 0·02 (S3) and 0·08 (S 4); $E_{5.7}^{His}$ 1·94, $E_{2.4}^{His}$ 1·37.

N$^{\alpha}$-Benzyloxycarbonylprolyl-N$^{\varepsilon}$-phenylacetyllysyl-glycine Amide (*III*)

A 4M-HBr solution in acetic acid (10 ml) was added to a solution of the protected dipeptide *II* (2·26 g) in acetic acid (10 ml). After standing for 30 min at room temperature, the dipeptide hydrobromide was precipitated with ether, collected on a filter, washed with ether and dried. The hydrobromide was dissolved in dimethylformamide (10 ml) and the solution adjusted to pH 10 with N-ethylpiperidine. A solution of benzyloxycarbonylproline (1·25 g) and 1-hydroxybenzotriazole (0·68 g) in dimethylformamide (10 ml) was added, the mixture was cooled to -10°C and treated with dicyclohexylcarbodiimide (1·1 g). After stirring for 1 h at -10°C and for 15 h at room temperature, the separated dicyclohexylurea was filtered off, dimethylformamide was evaporated and the residue triturated with 2% HCl. The insoluble portion was collected on a filter, washed with water, saturated solution of sodium hydrogen carbonate, again with water and finally with ether. Crystallization from methanol–ether afforded 2 g (72%) of the product, m.p. 197–199°C. $[\alpha]_D$ $-34{\cdot}5$ (*c* 0·2, dimethylformamide); R_F 0·53 (S1), 0·40 (S2), 0·50 (S3), 0·67 (S4), 0·77 (S7). For $C_{29}H_{37}N_5O_6$ (551·6) calculated: 63·14% C, 6·76%H, 12·69%; found: 63·41% C, 6·84% H, 12·56% N.

Removal of phenylacetyl group by penicillin amidohydrolase; A 4M solution of HBr in acetic acid (0·5 ml) was added to a solution of the tripeptide *III* (55 mg) in acetic acid. After 30 min at room temperature, the hydrobromide was precipitated with ether, washed with ether and dried; R_F 0·18 (S1), 0·50 (S4); $E_{5.7}^{His}$ 0·60, $E_{2.4}^{Gly}$ 0·98. The tripeptide-amide hydrobromide (2·3 mg) was dissolved in 0·1M phosphate buffer of pH 7·5 (260 µl) and treated with a penicillin amidohydrolase solution (135 µl; the enzyme solution was prepared as described above) and water (135 µl). The mixture was incubated at 37°C and samples were withdrawn after 90 min and 21 h. In both the samples only a single compound was found; R_F 0·08 (S1), 0·05 (S4), $E_{5.7}^{His}$ 1·44, $E_{2.4}^{His}$ 1·08.

S-Benzyl-β-mercaptopropionyl-tyrosyl-phenylalanyl-glutaminyl-asparaginyl-S-benzylcysteinyl-prolyl-N$^{\varepsilon}$-phenylacetyllysyl-glycine Amide (*IV*)

A 1·6M solution of hydrogen chloride in dioxane (0·33 ml) was added to a solution of S-β-benzylmercaptopropionyl-tyrosyl-phenylalanyl-glutaminyl-asparaginyl-S-benzylcysteine hydrazide[21] (239 mg) in dimethylformamide (2·5 ml). After cooling to -20°C, butyl nitrite (25·8 mg) in dimethylformamide (1 ml) was added with stirring, and the stirring was continued for 20 min at -20°C. The mixture was cooled to -40°C and adjusted to pH 7 with N-ethylpiperidine. A solution of prolyl-N$^{\varepsilon}$-phenylacetyllysyl-glycine amide hydrobromide (prepared from 207 mg of the protected tripeptide *III* by the above-described procedure) and N-ethylpiperidine (0·1ml) in dimethylformamide (2 ml) cooled to -40°C was then added. The mixture was then kept at 0°C for 3 days. The dimethylformamide was evaporated, the residue treated with 2% hydrochloric acid, the precipitate filtered, washed on the filter with water, saturated sodium hydrogen carbonate solution and water. Crystallization from aqueous dimethylformamide afforded 300 mg of the product which was further purified by gel filtration on Sephadex LH-20 in dimethylformamide; yield 280 mg (84%), m.p. 223–225°C. $[\alpha]_D$ $-42{\cdot}6°$ (*c* 0·2, dimethylformamide). Amino acid analysis: Tyr 1·00, Phe 1·05, Glu 1·01, Asp 0·99, Lys 0·96, Pro 1.00, Gly 1·00. R_F 0·53 (S1), 0·23 (S2), 0·60 (S3), 0·73 (S4), 0·67 (S7), 0·55 (S11). For $C_{68}H_{84}N_{12}O_{13}S_2.2\ H_2O$ (1 377) calculated: 59·28% C, 6·43% H, 12·20% N; found: 59·32% C, 6·35% H, 12·38% N.

Cyclic Disulfide of β-Mercaptopropionyl-tyrosyl-phenylalanyl-glutaminyl-asparaginyl-cysteinyl-prolyl-N$^{\varepsilon}$-phenylacetyllysyl-glycine Amide (*Va*)

The protected nonapeptide *IV* (100 mg) was dissolved in liquid ammonia (100 ml) and reduced with sodium under stirring until the blue coloration persisted for 15 s. The excess sodium was

destroyed with ammonium chloride, ammonia was removed under diminished pressure (water pump) and the dry residue dissolved in 0·01M-HCl (300 ml). The aqueous solution was extracted with ether and adjusted to pH 6·75 with 0·1M-NaOH. A 0·01M solution of $K_3Fe(CN)_6$ was added to the stirred mixture in the course of 1 h, the pH being kept at 6·75, the solution was adjusted to 3·8 with 0·01M-HCl and freeze-dried. The residue was purified by countercurrent distribution (50 transfers of the upper phase), the peak of distribution coefficient $K_{43} = 6{\cdot}14$ being concentrated to a small volume and freeze-dried. Further purification by gel filtration on Bio-Gel P-4 in 3M acetic acid yielded 22 mg of product, $[\alpha]_D$ −53·8° (*c* 0·36, 50% acetic acid). R_F 0·27 (S1), 0·15 (S2), 0·31 (S3), 0·73 (S4), 0·66 (S7). For $C_{54}H_{70}N_{12}O_{13}S_2.2\,H_2O$ (1 195) calculated: 54·25% C, 6·23% H, 14·06% N; found: 54·36% C, 6·27% H, 14·07% N.

Deamino-lysine-vasopressin (*Vb*)

A solution of the protected cyclic nonapeptide *Va* (18 mg) in 96% ethanol (0·7 ml) was diluted with water (6·3 ml). Penicillin aminohydrolase (150 mg) was dissolved in 0·1M phosphate buffer, pH 7 (5 ml), a small amount of undissolved compound was removed by centrifugation and the solution was added to the dissolved peptide. The mixture was incubated for 1 h at 37°C, cooled at room temperature and acidified with 0·01M-HCl (pH 3). The denaturated enzyme was removed by centrifugation and the solution desalted on a column of Amberlite CG-50 (15 × 1 cm), affording 25 mg of product which was purified by free-flow electrophoresis; yield 12·25 mg (74%) of pure compound, R_F 0·10 (S1), 0·40 (S4); $[\alpha]_D$ −89·9° (*c* 0·5, 1M acetic acid). Amino acid analysis: Lys 1·00, Asp 0·98, Glu 1·07, Pro 0·99, Gly 0·78, Tyr 1·02, Phe 1·14. For $C_{46}H_{64}N_{12}O_{12}S_2.C_2H_4O_2$ (1 101) calculated: 52·35% C, 6·22% H, 15·26% N; found: 52·08% C, 6·18% H, 15·32% N.

Pharmacological Methods

Uterotonic activity was determined on an isolated strip of rat uterus[29,30] placed in Mg^{++}-free solution. Galactogogic activity[31,32] was assayed on lactating rats (5 to 10 days after delivery). For determination of pressor activity we used nefrectomized rats[33].

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